THE CYTO-GENOTOXICITY EVALUATION OF BIOMEDICAL GRADE CHITOSAN ON PRIMARY KERATINOCYTE CULTURES AND ITS ROLE IN SCAR FORMATION PATHWAYS USING SKIN COCULTURE MODEL

by

LIM CHIN KEONG

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α	Alpha
&	And
~	Approximately
β	Beta
^{o}C	Degree Celsius
≥	Equal or more than
<	Less than
%	Percent
±	Plus or minus
®	Registered
™	Trademark
ACS	American Chemical Society
AIU	Arbitrary intensity unit
ALS	Alkali-labile sites
AMREC	Advanced Materials Research Centre
APS	Ammonium persulphate
BCG	Bacillus Calmette-Guerin
BHQ1	Black hole quencher-1
bp	Base pair
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy

Ca^{2+}	Ion calcium
$CaCl_2$	Calcium chloride
CAPS	3-cyclohexylamino-1-propanesulfonic acid
cDNA	Complementary deoxyribonucleic acid
cNHDF	Commercial normal human dermal fibroblasts
cNHEK	Commercial normal human epidermal keratinocytes
CO_2	Carbon dioxide
CPSRTs	Chitosan porous skin regenerating templates
CTGF	Connective tissue growth factor
cu	Caseinolytic unit
Ct	Cycle threshold
ddH_2O	Double distilled water
dH_2O	Distilled water
DKSFM	Defined keratinocyte serum free medium
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DPX	Di-n-butylPhthalate in Xylene
DSB	Double-stranded breaks
DTBP	Dimethyl 3-3,dithio-bis'propionimidate
E	Real-time polymerase chain reaction efficiency
ECM	Extracellular matrix/ extracellular matrices
EGF	Epidermal growth factor

ECVAM	European Center for the Validation of Alternative Methods
e.g.	*exempli gratia* (Example)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al.	*Et alii*
6-FAM	6-carboxyfluorescein
FBS	Fetal bovine serum
FDA	United States Food and Drug Administration
g	Earth's gravitational acceleration
g	Gram
gDNA	Genomic deoxyribonucleic acid
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H_2O	Water
HCl	Hydrochloric acid
HDF	Human dermal fibroblasts
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HKGS-V2	Human keratinocytes growth supplements
HMWC	High molecular weight chitosan
HRP	Horseradish peroxidase
HSP47	Heat shock protein 47
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin

ISO	International Standards Organization
JMHW	Japanese Ministry of Health and Welfare
kb	Kilobase
KCl	Potassium chloride
kDa	Kilodalton
KH_2PO_4	Potassium phosphate monobasic
L	Liter
LDPE	Low density polyethylene
LMAgarose	Low melting point agarose
LPS	Lipopolysaccharide
M	Molar
mA	Milliampere
MAPK	Mitogen-activated protein kinase
mg	Milligram
Mg^{2+}	Ion magnesium
$MgCl_2$	Magnesium chloride
mL	Milliliter
mM	Millimolar
mm^2	Millimeter square
MTT	3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl tetrazolium bromide
N	Normality
n	Number of samples/ replicates
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate

Na_2HPO_4-$7H_2O$	Sodium phosphate dibasic
NaOH	Sodium hydroxide
NC-bicarbonate	Neutralized chitosan by sodium bicarbonate
NC-drytech	Neutralized chitosan by drying technique
NC-etha	Neutralized chitosan solution by ethanol
ng	Nanogram
nm	Nanometer
nM	Nanomolar
Na-deoxycholate	Sodium deoxycholate
No.	Number
NO-CMC	*N, O*- carboxymethyl-chitosan
NP-40	Nonyl Phenoxylpolyethoxylethanol-40
NTC	No-template-control
O-C	Oligo-chitosan
OD	Optical density
PAI-1	Tissue plasminogen activator inhibitor
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDLC	Periodontal ligament cells
PMSF	Phenylmethanesulphonyl fluoride
PEG	Polyethylene glycol
PET	Polyethylene terephthalate
pg	Picogram
pH	Power of hydrogen

pHDF	Primary human dermal fibroblasts
pHEK	Primary human epidermal keratinocytes
pKHDF	Primary keloid human dermal fibroblasts
pKHEK	Primary keloid human epidermal keratinocytes
PMSF	Phenylmethanesulphonyl fluoride
pNHDF	Primary normal human dermal fibroblasts
pNHEK	Primary normal human epidermal keratinocytes
PSA	Penicillin, Streptomycin and Amphotericin
PVA	Polyvinyl alcohol
PVC	Polyvinyl chloride
PVDF	Polyvinylidene fluoride
R^2	Linear regression
Real-time PCR	Real-time polymerase chain reaction
QRT-PCR	Quantitative real-time polymerase chain reaction
rEGF	Recombinant epidermal growth factor
RFU	Relative fluorescence unit
RIPA	Radio-immuno precipitation assay
RNA	Ribonucleic acid
rpm	Round per minute
SCGE	Single cell gel electrophoresis
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy

SIRIM	Standards and Industrial Research Institute of Malaysia
SMAD	Similar to mothers against Decapentaplegic Drosophilia
SSB	Single-stranded breaks
TBS	Tris-buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF	Transforming growth factor
TMB	3.3', 5.5'-tetramethylbenzidine
TNF	Tumor necrosis factor
Tris-Cl	Tris base added with concentrated hydrochloric acid
USA	United States of America
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar
V	Volt
VEGF	Vascular endothelial growth factor
v/v	Volume by volume
WSC	Water-soluble chitosan
w/v	Weight by volume
YIGSR	Tyr-Ile-Gly-Ser-Arg, (Tyrosine-Isoleucine-Glycine-Serine-Arginine)

PENILAIAN KETOKSIKAN SECARA SITO-GENO DENGAN MENGGUNAKAN KITOSAN BERGRED BIOPERUBATAN PADA KULTUR KERATINOSIT ASAS DAN PERANANNYA DALAM LITAR PEMBENTUKAN PARUT DENGAN MENGGUNAKAN MODEL KO-KULTUR KULIT

ABSTRAK

Kitosan (β-1, 4—D-glukosamin) merupakan kitin yang kumpulan asetilnya telah disingkirkan dan ia mempunyai ciri-ciri biologi yang unggul dan berguna dalam rawatan luka. Tetapi, pengubahsuaian kitosan secara fizikal dan kimia akan menjejaskan bio-keserasiannya. Selain daripada itu, dilaporkan juga bahawa kitosan mempunyai kegunaan dalam pencegahan parut. Oleh itu dalam kajian ini, dengan menggunakan kultur normal keratinosit asas daripada epidermis manusia (pNHEK), bio-keserasian in vitro pada templat perbentukan kulit berpori kitosan (CPSRT) yang diubahsuai telah dinilai melalui analisis ketoksikan secara sito melalui ujian 3-(4, 5-dimetilthiazol-2-yl)-2, 5-difenil tetrazolium bromida (MTT), analisis ketoksikan secara geno dengan menggunakan ujian komet serta analisis rembesan sitokin keradangan keratinosit kulit [interleukin-1α (IL-1α), faktor tumor nekrosis-α (TNF-α) dan interleukin-8 (IL-8)]. Peranan CPSRT yang bio-serasi dalam pencegahan keloid telah diselidik dengan menggunakan ko-kultur keratinosit-fibroblast. Pengekspresan protein kolagen manusia berbentuk-I, III dan V telah diselidik dengan menggunakan analisis Western blot. Sementara itu, rembesan faktor pertumbuhan-β1 (TGF- β1) telah dianalisis dengan menggunakan ujian enzim-linked immunosorbent (ELISA) dan pengekspresan mRNA melalui molekul intraselular (SMAD 2, 3, 4 dan 7) juga telah dihitung dengan menggunakan analisis real-time PCR. Kultur keratinosit asas daripada epidermis manusia (pHEK) yang selnya berbentuk skuamosa telah disahkan dengan sitokeratin-6

(CK6) dan involukrin, manakala kultur fibroblast asas daripada dermis manusia (pHDF) telah dibuktikan dengan kewujudan heat shock protein 47 (HSP47) dan protein permukaan fibroblast (FSP). CPSRT-NC-bikarbonat telah meningkatkan kadar pertumbuhan sel dalam kultur pNHEK. Ia tidak mencetuskan kemusnahan DNA dan juga tidak meningkatkan respon sitokin keradangan secara in vitro. Ko-kultur keratinosit-fibroblast telah dipelihara dalam DKSFM:DMEM:F12 (2: 2: 1) sebagai satu medium yang dikongsi dalam ko-kultur. Kolagen bentuk-I adalah dominan dalam kultur normal fibroblast asas daripada dermis manusia (pNHDF) manakala kolagen bentuk-III pula banyak didapati dalam ko-kultur fibroblast daripada keloid dermis manusia (pKHDF). Kolagen bentuk-V berkemungkinan wujud dengan lebih banyak di dalam dermis yang mempunyai kolagen bentuk-I yang banyak. SMAD 2, SMAD 4 dan TGF-β1 telah didapati lebih banyak di dalam ko-kultur pKHDF berbanding dengan pNHDF. Ko-kultur yang wujud dengan keratinosit normal telah mengurangkan pengekspresan kolagen bentuk-III, SMAD 2, SMAD 4 dan TGF-β1. Rawatan dengan CPSRT-NC-bikarbonat meningkatkan lagi kesan itu. Kesimpulannya, CPSRT-NC-bikarbonat yang bio-serasi berhubungan dengan kultur normal keratinosit mempunyai bio-fungsi untuk mengurangkan fosforilasi TGF-β1 dalam kultur fibroblast yang berasal keloid. Ianya mungkin melalui peningkatan dalam pengekspresan SMAD 7 dan faktor-faktor larut CPSRT-NC-bikarbonat yang melembapkan reseptor TGF-β, lalu mengurangkan transkripsi ekstrasellular matriks (ECM) terutamanya kolagen bentuk-III, di mana ia berguna dalam pencegahan keloid.

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ABSTRACT

Chitosan (β-1, 4—D-glucosamine) is a deacetylated form of chitin with excellent biological properties useful in wound management. However, improvement of the physical and chemical properties of the chitosan will alter its biocompatibility. Additionally, chitosan has been reported to be useful in scar intervention. Therefore in this study, the *in vitro* biocompatibility of the modified chitosan porous skin regenerating templates (CPSRTs) has been evaluated based on cytotoxicity analysis by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay, genotoxicity by comet assay, and skin keratinocyte pro-inflammatory cytokine secretions [interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8)], using primary normal human epidermal keratinocyte (pNHEK) cultures. The most biocompatible CPSRT was then investigated for its role in keloid intervention using keratinocyte-fibroblast cocultures. The human collagen type-I, III and V protein expressions were assayed in Western blot analysis. Meanwhile, the secreted transforming growth factor-β1 (TGF-β1) was analyzed by enzyme-linked immunosorbent assay (ELISA), and the mRNA expressions of its intracellular signaling molecules (SMAD 2, 3, 4 and 7) were quantified using real-time PCR analysis. The established squamous-shaped primary human epidermal keratinocyte (pHEK) cultures have been verified using cytokeratin-6 (CK6) and involucrin, whereas spindle-shaped primary human dermal fibroblast (pHDF) cultures were tested for the presence of heat

shock protein 47 (HSP47) and fibroblast surface protein (FSP). Treatment with CPSRT-NC-bicarbonate enhanced the cell growth of pNHEK cultures. It did not induce DNA damage and did not provoke pro-inflammatory cytokine responses *in vitro*. Keratinocyte-fibroblast cocultures were maintained in DKSFM: DMEM: F12 (2: 2: 1) as a shared serum-free coculture medium. Collagen type-I is the dominant form in primary normal human dermal fibroblast (pNHDF) whereas collagen type-III is more abundant in primary keloid-derived human dermal fibroblast (pKHDF) cocultures. Collagen type-V may be present more in the skin dermal that is rich in collagen type-I. SMAD 2 and SMAD 4, as well as the TGF-β1 were expressed more in the pKHDF than pNHDF cocultures. Cocultures present with normal keratinocytes were observed to suppress collagen type-III, SMAD 2, SMAD 4 and TGF-β1 expressions. Treatment with CPSRT-NC-bicarbonate further enhanced this effect. In conclusion, the biocompatible CPSRT-NC-bicarbonate which was in association with normal-derived keratinocyte cultures exerted bio-functionality to decelerate TGF-β1 phosphorylation in the keloid-derived fibroblast culture, perhaps through the increased expressions of SMAD 7 and the soluble factors of CPSRT-NC-bicarbonate in deactivating the TGF-β receptors, thus reducing transcription of extracellular matrix (ECM) particularly collagen type-III, which can be useful in keloid intervention.

CHAPTER 1

INTRODUCTION

1.1 Research background

Wound healing is a process of restoration that is necessary for tissue repair, typically comprising a continuous sequence of inflammation and repair in which epithelial, endothelial, inflammatory cells, platelets and fibroblasts briefly interact to resume their normal functions. This healing process consists of four different and overlapping phases, namely: inflammation, granulation tissue formation, matrix remodeling and reepithelialization (Christine *et al*., 1999). The orderly sequence of healing events is regulated by various interplayed cytokines and growth factors (Ono *et al*., 1995). One of the ultimate goals in wound healing research involves finding ways for humans to heal *via* the regeneration of similar tissues. This could be accomplished *via* the invention and modification of a variety of dressing materials to facilitate proper wound management.

Chitosan is a partially deacetylated derivative of natural chitin, a primary structural polymer in arthropod exoskeletons. One of the present trends in biomedical research requires materials that are derived from nature as natural materials have been shown to exhibit better biocompatibility with humans. For example, chitosan's monomeric unit, *N*-acetylglucosamine, occurs in hyaluronic acid, an extracellular macromolecule that is important in wound repair. Studies on chitosan have been intensified since 1990 due to its low cytotoxicity, antimicrobial activity and excellent biodegradable properties in the human body, where these have contributed to the better biocompatibility of chitosan (Sashiwa *et al*., 1990; Shigemasa *et al*., 1994; Van *et al*., 2006; Prabharan and Mano, 2006). The potential of chitosan stems from

its cationic nature and high charge density in solution. An effective approach for developing a clinically applicable chitosan is to modify the surface of the material that already has excellent biofunctionality and bulk properties. However, blending technologies with various additives may cause cytotoxicity. Hence, the modified biomedical-grade of chitosan with various derivatives should be submitted for biocompatibility testing to promote the engineering of high-quality, biocompatible products for human wound management.

In the pre-commercialization steps of a biomedical-grade chitosan derivative as wound dressing, it is of great importance that the dressing be submitted to a number of *in vitro* pre-toxicity assays. *In vitro* toxicity examinations are preferable to *in vivo* tests since the newly developed dressing materials can be examined outside the body and the results are more reproducible, facilitating clinical evaluation of a medicinal product that can be replicated and estimated (Soheili *et al*., 2003; Cheong *et al*., 2008). Biocompatibility *in vitro* is useful to characterize cytotoxic effects of leachable materials, such as residual monomers, catalysts as well as polymer erosion and related factors (*e.g*. composition, molecular weight and polydispersity) which are suitable to monitor the quality and purity of the biodegradable biomedical-grade chitosan. Cytotoxicity assays measure only finite effects on cells during the first 12 to 72 hours after exposure to toxic substances. The host cells either recover from or succumb to their chemical injury. However, many biological reactions *in vivo* are not simply cytotoxic and are propagated beyond 24 hours. Examples are improper cells programmed apoptosis and necrosis due to deoxyribonucleic acid (DNA) damage; inflammatory and immune reactions that all resulted from non-biocompatible chitosan derivatives. Therefore, *in vitro* biocompatibility assessment for wound

management must consider cytocompatibility, genotoxicity and related pro-inflammatory cytokines expression for better *in vitro* biocompatibility evaluation.

Abnormal wound healing is related to improper fibroblasts activities in the wound bed that have commonly encountered in multiple pathologies ranging from diabetic ulcers, to fibrosis and scarring. Keloids render a form of abnormal wound healing affecting only human which involve persistent itchy pseudotumoral skin lesions expanding beyond edges of the original wounds and frequently recurring following surgical excision. The exuberant scarring of keloid leads to disfiguring and dysfunctional scars without any satisfactory treatment until today. Hence, by understanding the keloid formation pathway may serve as a corner stone for the treatment. Unluckily, there are still no keloid studies using established animal model (Xia *et al*., 2004). Alternatively, keloid studies are mostly performed using an *in vitro* model (Uitto *et al*., 1985; Smith *et al*., 1999; Higham *et al*., 2002; Xia *et al*., 2004; Naitoh *et al*., 2005).

Wound healing is a complex physiological response that involves various interactions between epithelial and mesenchymal. Many earlier *in vitro* studies on keloid pathogenesis were largely on the use of keloid fibroblasts simply because it mainly produces collagen and extracellular matrix (ECM) that form the bulk of keloid tissue. Recently, evidence has indicated that autocrine, paracrine and endocrine epithelial-mesenchymal interactions may be involved in normal skin homeostasis, growth and differentiation (Maas-Szabowski *et al*., 1999; Higham *et al*., 2005; Amjad *et al*., 2007). Therefore, the application of *in vitro* coculture system may serve as a useful tool to elucidate the pathogenesis of keloid. Besides, biochemical and ultrastructural studies of the engineered skin *via* cultures or cocultures had also shown the similarities in metabolic and differentiation program

compared with human skin (Asbil *et al*., 2000; Robinson *et al*., 2000; Wagner *et al*., 2001). Human skin tissue culture may provide a controlled yet realistic model to study the molecular mechanisms of barrier disruption and identify the involvement of genes in a disease development such as scar formation. By using a specifically made coculture inserts, that is a membrane with certain pore sizes used to culture one type of cell line while the other cell line on another plastic dishes. The combination of both inserts will imitate the *in vivo*-like interactions in which the skin keratinocytes are separated from dermal mesenchymal cells by a complex ECM, the basement membrane, thus rendering cell-cell contact-mediated mechanisms less probable.

Fibroblasts are the primary cell type that functions in the organization of the ECM following of skin injuries. To restore the barrier function of the skin as quickly as possible, closure of skin wounds and replacement of missing skin tissues occur rapidly in adult skin by some mechanisms that lead to scarring in which overproduction of collagen bundles renders significant keloid pathology. Collagen type-I, III and V constitutes a large portion in the keloid ECM (Ala-Kokko *et al*., 1987). In accordance with the clinical observation that delayed reepithelialization is associated with the scar formation, *in vitro* investigations have demonstrated that keratinocytes decrease fibroblasts expression of collagen and pro-fibrotic factors (Garner, 1998; Le Poole and Boyce, 1999). Some have reported that the chitosan may be potentially used in scar prevention (Wang *et al*., 1998; Paul and Sharma, 2004; Yang *et al*., 2006; Delorino and Cresidio, 2009). Among the TGF-β isoforms, TGF-β1 is one of the most reported and important stimulators of collagen synthesis. It affects the ECM not only by stimulating collagen synthesis but also by preventing its breakdown (Yang *et al*., 1997; Szulgit *et al*., 2002; Quan *et al*., 2004). TGF-β1 is a pro-fibrotic factor that increases the formation of granulation tissue and stimulates

wound contraction besides being a negative regulator of inflammation and responsible in the switching of the inflammatory phase to the proliferation phase in wound healing. Similar to mothers against Decapentaplegic Drosophilia (SMAD) proteins function as intracellular signaling mediators of TGF-β family members and can be subdivided into receptor-regulated SMADs (R-SMADs), common mediator SMADs, and inhibitory SMADs (Ten and Hill, 2004). Kopp *et al*. (2005) proposed the relationship of TGF-β/ SMADs in human fibroproliferative disorders and elucidated that autocrine TGF-β/ SMADs signaling is involved in contractility and matrix gene expression of the fibroproliferative diseases.

Therefore, in this study, primary normal human epidermal keratinocytes (pNHEK) was implemented in the cytotoxicity pre-evaluation of newly developed chitosan porous skin regenerating templates (CPSRTs). Those contributed more than 70% of cell viability were then chosen for genotoxicity assay using alkaline single cell gel electrophoresis (SCGE) or comet assay. Additionally, their potential to induce skin irritation or inflammatory responses was assessed by quantitatively examining the secretion of pro-inflammatory cytokines (IL-1α, TNF-α and IL-8) in the pNHEK cultures. Keratinocyte-fibroblast coculture model was also constructed to study the epithelial-mesenchymal interactions of normal and keloid-derived human skin tissues, with regard to the effectiveness of CPSRTs on the keloid intervention, by elucidating the expressions of type-I, III and V collagen and its response in TGF-β1/ SMADs signaling pathway.

1.2 Literature review

1.2.1 Structure and function of human skin

Skin is the largest organ in the human body besides commonly known as a vital outpost of the nervous system and is the closest contact with the outside world. It naturally provides a protective covering layer for the other tissues in the body, acting as a barrier to micro-organisms, water and chemicals, regulating body temperature while still maintaining the water content of the body, removing waste products from the body and providing sensory information. In addition, the skin produces Vitamin D in response to sunlight (Brad Spellberg, 2000). Generally, human skin is composed of three structurally and functionally integrated layers namely the outer epidermis, the dermis and the inner hypodermis (Haake and Holbrook, 2001).

The human skin epidermis consists mainly of keratinocytes that form a stratified squamous epithelium (Eckert *et al*., 1997). Its thickness is about 0.2 mm on average and this thickness varies depending on the location on the body. Furthermore, the thickness also varies according to the volume of water that the epidermis holds. Epidermal layer itself is further divided under skin cell differentiation process into four main layers that are stratum basal, stratum spinous, stratum granular and stratum corneum. These layers render to different stages of cell maturation. Keratinocytes at the basal layer progressively differentiate upward into cells with morphologically bigger and flatter-shaped meanwhile loosing proliferative ability (Jetten and Harvat, 1997). Epidermal population of stem cells that are confined to the basal cell layer of the epidermis will constitutively provide the epidermis with its regenerative capacity. Cells from the basal layer migrate and replicate during reepithelialization of wound healing (Haake and Holbrook, 2001).

Basal layer keratinocytes are columnar in shape and adhere with the underlying basement membrane through the hemidesmosomes. In the outermost layer which is stratum corneum, cells are cornified, lacking nuclei and organelles besides composed primarily of keratins. The cornified cells desquamate in order to balance the proliferation in the basal layer at the turnover rate of approximately 21 days. There are also small portions of cells residing in the human skin epidermal namely pigment-producing melanocytes, antigen-presenting Langerhans cells and neuroendocrine Merkel cells. The epidermal appendages (hair follicles, apocrine glands, sweat glands and sebaceous glands) extend into the human skin dermis. In superficial wounds where most of the dermis is intact and viable, the cells in the epidermal appendages proliferate and regenerate the epidermis (Fairley, 2001).

The dermis is the second layer of human skin, beneath the epidermal layer. This layer is much thicker than the epidermis ranging from 1 to 4 mm. The dermis is structurally organized into the uppermost papillary and the innermost reticular dermis. At the dermal-epidermal junction, the dermal papillae interact with extensions of the epidermis called rete pegs. The sub-papillary plexus which is lying as horizontally vessels constitutes the boundary between the papillary and reticular regions besides extending capillaries toward the skin epidermis (Haake and Holbrook, 2001). The skin dermis has few cells and is mainly composed of connective tissue and extracellular matrix (ECM) (Lee *et al*., 2006). The essential dermal cell type is fibroblast which is responsible for the production and maintenance of the structural elements of skin. These elements are collagen and elastin which are the major fibrous components in the reticular dermis, combining with non-fibrous substances such as glycosaminoglycans (GAGs) to form ECM (Butler *et al*., 1999). Fibroblasts are more abundant in the papillary dermis and have

higher proliferative and metabolic activity at this location. Collagen is the major constituent accounting approximately 75% of the dry weight of human skin. There are several types of collagen present in mammalian skins. Collagen type-I, III and V commonly are found in human in which normal skin dermal consists approximately 80% of collagen type-I. The connective tissues of the dermis give the skin its pliability, elasticity, tensile strength and protect the body from mechanical injuries (Brown *et al.*, 1994). During deep wound healing, skin dermal fibroblasts are responsible for the formation of scar tissue accompanied with activated immune response and reparative processes.

Hypodermis is a layer underneath the dermis which is an abrupt transition from the reticular dermis. This layer is rich in adipose tissue. However, skin dermis and hypodermis are integrated through nerve and vascular networks as well as epidermal appendages. The adipocytes are organized into lobules separated by septa of connective tissues that contain nerves, vessels and lymphatics (Wajchenberg, 2002). This hypodermis insulates the human body, provides energy and allows for mobility of the skin on underlying structures. Adipose tissue that is underlying the hypodermis serves as an energy storage, in which is virtually limitless *via* lipogenesis over lipolysis and pre-adipocyte replication and differentiation (Prins and O'Rahilly, 1997).

1.2.2 Cutaneous wound healing

Wound is defined as the disruption of the anatomic structures and their functions in any body parts. This can be due to a simple cut or burns. Wounds can be generally classified as wounds without tissue loss (*e.g*. in surgery) and wounds with tissue loss such as burn wounds, wounds due to trauma, abrasions or as secondary events in chronic ailments (*e.g*. venous stasis, diabetic ulcers and iatrogenic wounds such as skin graft donor sites and derma-brasions) (Willi and Chandra, 2004). In contrast, wound healing is a process of restoration by which tissue repair takes place and usually comprises of a continuous sequence of inflammation and reparation in which epithelial, endothelial, inflammatory cells, platelets and fibroblasts briefly interact to restore a semblance of their usual discipline and resume their normal functions. The orderly sequence of healing events are accomplished and regulated by cytokines and growth factors (Ono *et al*., 1995). Wound healing is a process involving generally three phases namely inflammation, proliferation and tissue remodeling. Soon after the elimination of macrophage that appears during the inflammatory phase, wound healing is then impeded and the tensile strength of the scar is diminished (Leibovich and Ross, 1975).

1.2.2.1 Inflammation phase

Cutaneous injury disrupts primarily the blood vessels and lymphatics. An initial five to ten minutes period of vasoconstriction is then followed by more persistent vasodilatation. This presents itself as erythema, swelling, warmth and is often accompanied with pain. Blood components are recruited into wound cavity. Endothelial cells retract and loose their attachments with adjoining cells, exposing subendothelial factor VII related Von Willebrand factor and fibrillar collagen in the

injured tissue. Platelets adhere to these surfaces to form platelet plug, with association of fibrin clot provides a protective wound matrix. The provisional matrix composed of fibrin and small amounts of vitronectin, fibronectin and thrombospondin. Platelets release inflammatory mediators that together with bacterial peptides and products of matrix degradation attract leukocytes to the wound. Neutrophil migrations engulf debris and microorganisms providing the first line of defense against infection for a few days. If acute inflammation phase persists due to wound hypoxia, infection, nutritional deficiencies or other factors related to one's immune response, it can interfere with the late inflammatory phase (Stadelmann *et al*., 1998). Macrophages replace neutrophils towards the end of the inflammatory phase. Macrophages release several growth factors that stimulate the migration of proliferation of fibroblasts as well as the production of ECM. Thrombocytopenia decreases inflammatory activity during wound healing but it does not impede cutaneous tissue repair (Szpaderska *et al*., 2003).

1.2.2.2 Proliferation phase

The subsequent proliferation phase is dominated by the formation of granulation tissue and reepithelialization. Chemotactic and growth factors released from platelets and macrophages stimulate the migration and activation of wound fibroblasts that produce a variety of substances such as glycosaminoglycans (mainly hyaluronic acid, chondroitin-4-sulfate, dermatan sulfate and heparin sulfate) and collagen (Stadelmann *et al*., 1998). This amorphous and gel-like connective tissue matrix is necessary for cell migration. Reepithelialization begins where the basal keratinocytes flatten and detach from the basement membrane and migrate over the wound matrix. There is also evidence that suprabasal keratinocytes take part in the

reepithelialization (Laplante *et al*., 2001). Fibronectin, laminin 5 and collagen type I that reside in the ECM are known to assist the keratinocytes migration while collagen type IV, thrombospondin and laminin may inhibit the migration (Frank and Carter, 2004; Woodley *et al*., 1991). Antimicrobial peptide LL37 has also been regarded as one of the factors involved in the reepithelialization (Heilborn *et al*., 2003). Matrix degrading enzymes are highly increased in wound healing and facilitate the migration of keratinocytes (Pilcher *et al*., 1999).

During the end of migratory phase of keratinocytes and where the components of basement membrane begin to aggregate, the migration is terminated leading subsequently to the proliferation and differentiation of keratinocytes to reconstitute the epidermis. Epidermal reconstitution requires migration, proliferation, differentiation and stratification of keratinocytes in cutaneous wound healing (Laplante *et a*l., 2001). Interactions between skin keratinocytes and fibroblasts in regulating basement membrane formation, cell proliferation and differentiation *in vitro*, may probably occur *in vivo* (Smola *et al*., 1998). Dermal fibroblasts may promote reepithelialization by secreting keratinocyte growth factor (KGF), insulin growth factor-1 (IGF-1) and the epidermal growth factor family (Haase *et al*., 2003; Werner and Smola, 2001). There is accumulating evidence for a possible role of interleukin-1 in reepithelialization (Werner and Smola, 2001; Maas-Szabowski *et al*., 2000). Thus, hypertrophic scar formation in wounds due to delayed reepithelialization suggests a role for keratinocytes in diminishing the production of ECM by fibroblasts.

The dermal component of the wound heals through matrix synthesis (fibroplasia) and wound contraction, which is a centripetal movement of the edges of a full thickness wound in order to facilitate closure of the defect (Grillo, 1964).

Blood vessels are formed in the process of angiogenesis. Collagen synthesis and cross-linkage is responsible for vascular integrity and strength of new capillary beds. Improper cross-linkage of collagen fibers has been reported in nonspecific post-operative bleeding in patients with normal coagulation parameters (Blee *et al*., 2002). Fibroblasts and endothelial cells proliferate and start to migrate into the wound about 48 hours after injury in which fibroblasts form the new connective tissue while endothelial cells give rise to new vessels. Some fibroblasts differentiate into myofibroblasts that are characteristically similar to both smooth muscle cells and fibroblasts. Singer (1979) has described such a specialized structure as the 'fibronexus' which associates across the cytoplasmic membrane of the myofibroblasts of intracellular actin microfilaments and extracellular fibronectin fibers. Therefore, contraction of the actin filaments within the myofibroblasts will lead to the fibronexus to transmit this force to the surrounding matrix and mediate the clinical phenomenon of wound contraction that aids in closing the wound.

1.2.2.3 Tissue remodeling

Tissue remodeling is the final stage of cutaneous wound healing which continues for months after injury. The events in remodeling are responsible for the increase in tensile strength, decrease in erythema and formation of scar tissue bulk with the final appearance of the healed scar. As collagen is laid down, fibronectin gradually disappears. The nonsulfated glycosaminoglycans and hyaluronic acid are replaced by more resilient proteoglycans such as chondroitin-4-sulfate. Additionally, re-absorption of water from the scar allows the collagen fibers and other matrix components to lie closer together leading to an increase in the tensile strength. A

matrix rich in collagen type-I replaces the provisional matrix and granulation tissue which are rich in fibronectin and collagen type-III (Guo *et al*., 1990).

1.2.3 Chitosan as wound dressing

Wound dressings are generally classified based on its nature of action as passive products, interactive products and bioactive products. Traditional dressings such as gauze and tulle dressings that account for the largest market segment are known as passive products. Polymeric films and forms which are mostly transparent, permeable to water vapor and oxygen but impermeable to bacteria are commonly recognized as interactive products. Bioactive dressings are important in delivering substances in wound healing by which the delivery of bioactive compounds or dressings is constructed from material having endogenous activity. These materials include proteoglycans, collagen, non-collagenous proteins, alginates or chitosan (Willi and Chandra, 2004).

Chitosan is an *N*-deacetylated derivative bio-product of chitin and is found in the cell walls of some fungi (*e.g. Basidiomycetes spp.*), arthropods and marine invertebrates. Chitosan is usually produced from chitin by alkali treatment (Roller and Covill, 1999). Commercial chitosan is commonly deacetylated greater than 70% of deacetylation with molecular weights ranging between 100 000 to 1.2 million dalton (Onsoyen and Skaugrud, 1990). Chitosan is polycationic at pH < 6 and interacts readily with negatively charged substances such as protein, anionic polysaccharides (*e.g.* alginate, carragenan), fatty acids, bile acids and phospholipids due to the high density of amino groups present in the polymer (Muzzarelli, 1996). Their unique properties include polyoxysalt formation, the ability to form films, chelate metal ions and optical structural characteristics (Larry, 1998). Most of the current polymers are synthetic materials. Their biocompatibility and biodegradability are much more limited than chitosan and their derivatives. However, these naturally abundant materials also exhibit a limited reactivity and processing ability (Mass *et*

al., 1998). Chitosan's positive surface charge enables it to effectively support cell growth (Zileinski and Acbischer, 1994).

Chitosan is well known for its haemostatic properties to prevent excessive bleeding, while its biological activities can mediate macrophage function to secrete numerous enzymes (*e.g*. collagenase) and cytokines [*e.g*. interleukins, tumor necrosis factor (TNF)] in wound healing processes. Its biological properties include bacteriostatic and fungistatic which are particularly useful for wound treatment (Majeti and Ravi, 2000). It appears that chitosan which has structural characteristics similar to glycosamino glycans, could be considered for developing such substratum for skin replacement. Yannas *et al*. (1982) proposed a design of artificial skin for long term chronic use, focusing on a non-antigenic membrane in which can perform as a biodegradable template for the synthesis of neodermal tissue. According to Yao *et al*. (1994), chitosan can be used as an artificial skin in accelerating wound and ulcer healing besides serving as a biocompatible vehicle for sustained release of drug. Recently, application of chitosan has been given serious research attention due to its potentially important renewable resource that is non-toxic and biodegradable. Malette *et al*. (1986) studied the effect of treatment with chitosan and saline solution on healing and fibroplasia of wounds made by scalpel insertions in skin and subcutaneous tissue in the abdominal surface of dogs. It has been suggested that chitosan may be used to inhibit fibroplasia in wound healing and to promote tissue growth and differentiation in tissue culture (Muzzarelli *et al*., 1997). Some have reported that the chitosan may be potentially used in scar prevention (Wang *et al*., 1998; Paul and Sharma, 2004; Yang *et al*., 2006). Delorino and Cresidio (2009) concluded that by using 10% lactic acid for decalcification and 1M NaOH for deproteination chitosan is effective as active ingredient in scar remover cream.

Schmidt *et al*. (1993) have also reported the properties of chitosan in preventing overgrowth of fibroblasts besides being cytocompatible (Risbud *et al*., 2000) and blood biocompatible (Risbud and Bhat, 2001). The excellent biological properties of chitosan can be potentially improved with a variety of additional chemicals such as polyethylene glycol and carboxymethyl, *N*-acyl groups in order to produce biocompatible chitosan derivatives for use as wound dressing (Zhang *et al*., 2002). Chitosan-gelatin sponge wound dressing had shown better antibacterial effect by vacuum dryness on *Escherichia coli* K88 than that of penicillin besides having the superior healing effect to that by 0.2% (v/v) ethacridine. Additionally, chitosan-gelatin sponge assisted the wound contraction and shortened the wound healing time compared with sterile Vaseline gauze (Deng *et al*., 2007). Clinical assessment was performed using a Hyphecan cap (1-4,2-acetamide-deoxy-β-D-glucan), as a biological fingertip dressing and was proven to be effective in achieving a good functional and cosmetic result within a relatively short time, aside from being comfortable during conventional dressing changes (Halim *et al*., 1996).

1.2.4 Biocompatibility *in vitro* of biomedical-grade chitosan

Biocompatibility of a biomedical-grade chitosan refers to the quality of the material that does not have toxic or injurious effects on biological systems. Biocompatibility of any biomaterials is necessary to be tested for their strength, esthetics and clinical manipulation. Current regulations in accordance with the United States Food and Drug Administration (FDA), the International Organization for Standardization (ISO) and the Japanese Ministry of Health and Welfare (JMHW) require that manufacturers conduct adequate safety testing of their finished devices through pre-clinical and clinical phases as part of the regulatory clearance process. Toxicity induced by a chemical substance or drug can exert limitation on dose and duration of treatment besides adversely affecting patients' quality of life and may be life threatening. Side effects together with tissue toxicity will lead to long term effects such as alteration to the immune system and development of malignancies probably due to genetic damage induced by a certain kind of drug treatment (Paola *et al*., 2002). In the pre-commercialization steps of any biomaterial, it is of importance to undergo a number of pre-toxicity tests *in vitro* or *in vivo*. *In vitro* toxicity examinations are more favorable over the *in vivo* tests since the newly developed biomaterials can be examined outside the body and the results are more reproducible. Thus, preliminary *in vitro* tests are always being carried out to pre-screen and characterize the potentially harmful effects of a newly synthesized dressing material before it is clinically used on humans (Zhang *et al*., 2002). However, *in vitro* tests may not necessarily fully represent the *in vivo* test whereby differences in sensitivities had been observed between primary and established cell lines (Geurtsen *et al*., 1998; Lovschall *et al*., 2002). Various *in vitro* cell culture systems have been applied to investigate and evaluate the cellular processes, such as fibroblast and

keratinocyte proliferation and migration response to the growth factors that are present in a wound (Schreier *et al*., 1993; Cha *et al*., 1996; Kawada *et al*., 1997). Such *in vitro* models simplify and enable chemicals and biomaterials to be assessed for their potential, at least in a preliminary way, to promote wound repair by stimulating cell proliferation and for their biocompatibility.

Chitosan is a cationic polymer having an amino group in its chemical structure. Its anti-tumor, anti-microbial and anti-inflammatory properties can be largely attributed to the cationic structure. The *N*-acetylglucosamine moiety in chitosan is structurally similar to glycosaminoglycans (GAGs), heparin, chondroitin sulphate and hyaluronic acid in which they are biocompatible, and have specific interactions with various growth factors, receptors and adhesion proteins besides being the biologically important mucopolysaccharides in all mammals. Therefore, the analogous structure in chitosan may also exert similar bioactivity and biocompatibility (Li *et al*., 1992; Suh and Matthew, 2000). Additionally, chitosan can function as a bridge to improve the mechanical strength of collagen-based scaffolds owing to the large number of amino groups in its molecular chain (Taravel and Domard, 1996). Lee *et al*. (2002) performed trypan blue dye exclusion cytotoxicity assay on tumor cell lines, after increased positive charge density of chitosan by dialkylaminoalkylation and reductive amination followed by quaternization. Amino groups of chitosan attached with biologically active moieties such as vanillin, *p*-hydroxybenzaldehyde, *p*-chlorobenzaldehyde, anisaldehyde, methyl 4-hydroxybenzoate, methyl 2,4-dihydroxybenzoate, propyl 3,4,5-trihydroxybenzoate and 2-hydroxymethylbenzoate can promote anti-microbial ability (Kenaway *et al*., 2005).

A thorough understanding of cell and protein interactions with artificial surfaces is of importance to design suitable implant surfaces and substrates. The surface properties of newly synthesized biomedical-grade chitosan derivatives, including surface composition, wettability, domain composition, surface oxidation, surface charge and morphology, may influence protein adsorption and subsequently, the cellular responses to biomaterial implants. Though it has once been suggested that surfaces modified with polyethylene glycol (PEG) may limit protein adsorption and cell adhesion, the results for PEG blended with chitosan increased both adsorption and cell adhesion (Saneinejad and Shoichet, 1998). Peng *et al*. (2006) demonstrated that chitosan-collagen composites appeared as a biocompatible biomaterial for periodontal tissue engineering. Zhang *et al*. (2002) examined the biocompatibility of newly synthesized chitosan-PEG by growing and observing the morphological changes of 3T3 embryonic mouse fibroblasts and osteogenesis nodule cell suspension in culture. Cytotoxicity of chitosan in three dimensional fiber meshes was well determined with scanning electron microscopy (SEM) and cell morphological observation on the use of mouse lung fibroblast (L929) (Tuzlakoglu *et al*., 2004). Recent studies have been focused on the development of antibacterial surfaces of biomedical-grade chitosan derivatives to attain high functionality and high-value products. Huh *et al*. (2001) prepared chitosan-grafted poly(ethylene terepthalate) (PET) and quaternized chitosan-grafted PET, both showed high growth inhibition in the range of 75% to 86% and still retained 48% to 58% bacterial growth inhibition after laundering. But, higher antibacterial activity of a wound dressing can retard cell growth in culture. Laminin is known to be involved in metastasis of tumor cells. A peptide containing the Tyrosine-Isoleucine-Glycine-Serine-Arginine [Tyr-Ile-Gly-Ser-Arg (YIGSR)] sequence, corresponding to a partial sequence of laminin,

inhibited angiogenesis and therefore depressed tumor growth. Nishiyama *et al*. (2000) prepared YIGSR-chitosan conjugate which proved to have higher inhibitory activity against experimental lung metastasis of B16BL6 melanoma cells in mice than did the parent peptide. According to Koo *et al*. (2002), water-soluble chitosan (WSC) significantly protected the cultured cells from serum starvation-induced cellular rounding up and from serum starvation-induced cell death through p53 activation as tested by flow-cytometry and western blot. From these results, it appears that WSC may prevent serum starvation-induced apoptosis of human astrocytes *via* p53 inactivation. Cross-linking will increase the toxicity of chitosan scaffold leachate to fibroblast cells. However, the chitosan crosslinked with dimethyl 3-3,dithio-bis'propionimidate (DTBP) permitted cell proliferation after three days in culture (Adekogbe and Ghanem, 2005).

Denuziere *et al*. (1998) examined the cytocompatibility of films of chitosan and chitosan associated with GAGs in human skin keratinocytes and found that the cell growth was consistently to approximate 60% of that of controls. Highly deacetylated chitosans can modulate human skin cell mitogenesis *in vitro*. Cell compatibility of *N*-sulfoalkylchitosan derivatives with human dermal fibroblasts (HDFs) was dependent on the concentration of sulfonate moieties in which higher concentration led to decrease in cell compatibility (Jung *et al*., 2007). Additionally, Zainol *et al*. (2008) described the usefulness of chitosan to be developed into porous bilayer membrane as a skin regenerating template for treatment of third degree burn, and was also proven to be cytocompatible for skin fibroblasts as the cultured cells were noticed to proliferate on the chitosan membrane. Analysis of their effects on cells in culture may be useful as a screening tool for their potential activity *in vivo* as

wound healing agents. But, it is important to select appropriate strains of cells for use in the *in vitro* biocompatibility screening (Howling *et al*., 2001).

1.2.4.1 Cytocompatibility assessment *in vitro*

One of the most basic and important parameters in the *in vitro* measurement of a medicinal product's biocompatibility is cytocompatibility *in vitro*, which measures the qualitative and quantitative aspects of the impact of a medicinal product with regard to the viability of target cultured cells. There are a number of assays which are capable of evaluating the activation of biological processes (*e.g*. inflammation, immune reactions and mutagenesis) which require longer periods of tissue reaction to materials compared with cytotoxic reactions. Traditional assays have measured cytotoxicity *in vitro* by means of either an end-stage event (*e.g*. permeability of cytoplasmic membranes of dead and dying cells, or some metabolic parameters such as cell division or an enzymatic reaction) (Hanks *et al*., 1996). Cytotoxicity assays are commonly used to indicate the only finite effects on cells during the exposure to toxic substances at multiple concentrations and time course parameters. The colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay is a simple assay which is selected to determine detrimental intracellular effects on mitochondria and metabolic activity based on the selective ability of viable cells to reduce tetrazolium bromide into purple formazan crystals which are only soluble in organic solvents. The quantity of formazan crystal formed depends on intact metabolic activity and is frequently used for screening of cytotoxicity (Mossman, 1983). *In vitro* biocompatibility evaluation of biomedical-grade oligo-chitosan (O-C) and *N, O*- carboxymethyl-chitosan (NO-CMC) derivatives (O-C 1%, O-C 5%, NO-CMC 1% and NO-CMC 5%) correlated well

with *in vivo* study in which O-C 1% being the most cytocompatible using MTT cell viability assay. The experiment was carried out as described by the International Standards Organization (ISO), with some modifications (Lim *et al*., 2007). The MTT assessment of cytocompatibility was adequately good and the results were easily interpreted, besides being cost effective (Mori *et al*., 1997; Chellat *et a*l., 2000; Janvikul *et al*., 2005; Mei *et al*., 2005; Neamnark *et al*., 2007).

Appropriate target cell to be cultured should be in accordance with the end usage of a newly synthesized biomaterial. Je *et al*. (2006) suggested that chitosan derivatives in their studies were specific to tumor cell lines [HeLa (cervix cancer), HT108 (human fibrosarcoma) and A549 (lung cancer)]. Several transformed keratinocyte culture models have been used to study carcinogenesis, differentiation, apoptosis and cell cycle regulation (Yuspa *et al*., 1994). L929 mouse fibroblast cell line was often applied to evaluate cytotoxicity *in vitro* of various chitosan derivatives (Mori *et al*., 1997; Janvikul *et al*., 2005). Zange *et al*. (1998) implemented the MTT assay to pre-screen the cytotoxicity of poly(L-lactic-co-glycolic acid) attached with poly(ethylene oxide), namely ABA triblocks by using L929 mouse fibroblast cell lines and proved the better sensitivity of *in vitro* model for the cytotoxicity evaluation than studies in experimental animal model. Peng *et al*. (2006) observed the cell viability of human periodontal ligament cells (PDLCs) seeded within the chitosan-collagen scaffolds by using MTT assay and proved that chitosan-collagen composites are promising scaffold materials for periodontal tissue engineering. Others such as F1000 embryonic skin and muscle fibroblasts (Chung *et al*., 1994) were also implemented for the cytotoxicity evaluation. Cytotoxicity of chitosan with various degrees of *N*-deacetylation to form aminoethyl-chitosan, dimetylaminoethy-chitosan and diethylaminoethyl-chitosan, was examined with MTT on tumor cell

lines and concluded that cytotoxic effect may be caused by cationic charge of the chitosan derivatives (Je *et al*., 2006). Howling *et al.* (2001) had successfully demonstrated the use of human skin dermal fibroblasts and epidermal keratinocytes to evaluate the effect of chitosan with various deacetylation levels and polymer lengths *in vitro*. Jung *et al*. (2007) explored the cytocompatibility of newly synthesized *N*-sulfopropylchitosan derivatives by using MTT assay on human dermal fibroblasts (HDF) culture and suggested the usefulness of MTT assay together with the use of other cell types in assessing the cytocompatibility of chitosan derivatives. HDFs cultures were also implemented along with MTT cell viability assay to examine the cell proliferation within the newly developed chitosan-based porous scaffold (Lim *et al*., 2007).

1.2.4.2 Genotoxicity assessment *in vitro*

Although measures of a medical product's biocompatibility have largely been reported in terms of irritation, sensitization, and systemic toxicity, there is a growing concern that devices or biomaterial extracts may exert some genotoxic effects. Thus, any attempts to assess the safety of a device intended for intimate body contact or permanent implantation would be incomplete without testing for the presence of toxins that might exert an effect on the genetic material of cells. In its set of harmonized standards for the biological evaluation of medical devices, the ISO has outlined the need for such genotoxicity testing in ISO 10993-3: "Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicity."

A number of techniques for detecting deoxyribonucleic acid (DNA) damage as opposed to the biological effects (*e.g*. Ames test, micronuclei, mutations, structural chromosomal aberrations) (Ames *et al*., 1973; Savage, 1976; Ishidate,

1979; Maron and Ames, 1983) that result from DNA damage have been used to identify substances with genotoxic activity. According to Muramatsu *et al*. (2004), they did not detect any genotoxicity *via* Ames test and chromosome aberration assay, performed on Chinese hamster lung fibroblasts with 5mg/ml (maximum concentration) of beta-tricalcium phosphate/carboxymethyl-chitin. Abou Sereih *et al*. (2007) reported that their chitosan with concentrations of 3.00 and 4.50 mg/ml have induced mutation on *Trichoderma*. However, those techniques have some shortcomings (*e.g*. time consuming, requirement for proliferating cell populations) (Fernandez and Ferrao, 1999; Giri *et al*., 1999; Ikken *et al*., 1999; Umbuzeiro *et al*., 1999).

Until recently, a more useful approach for assessing DNA damage is the single-cell gel electrophoresis (SCGE) or comet assay. The terms “Comet” is used to identify the individual cell DNA migration patterns produced. According to Ostling and Johanson (1984), they were the first to develop a microgel electrophoresis technique for detecting DNA damage at the single cell level. In their technique, cells were embedded in agarose and placed on a microscope slide. The cells were lysed by detergents and high salt concentration to liberate the DNA. The liberated DNA was then electrophoresed under neutral conditions. Therefore, cells with an increased frequency of DNA double strand breaks (DSB) displayed increased migration of DNA toward the anode. The migrating DNA was then measured quantitatively by staining with ethidium bromide using a microscope photometer. The neutral condition used in the comet assay detects only DSB of a cell. Subsequently, Singh *et al*. (1988) introduced a microgel technique involving electrophoresis under alkaline (pH >13) conditions for detecting DNA single strand breaks (SSB) and alkali-labile sites (ALS) in single cells. Singh (2000) mentioned that the alkaline comet assay can